A PAPER CHROMATOGRAPHIC SURVEY OF FREE AMINO ACIDS AND RELATED COMPOUNDS IN THE PROTEACEAE

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Abstract

Fifty species of the Proteaceae, belonging to the genera *Brabeium*, *Paranomus*, *Serruria*, *Mimetes*, *Faurea*, *Protea*, *Leucospermum*, *Leucadendron* and *Aulax* were analysed for free ninhydrin-positive compounds by means of paper chromatography. Altogether 38 compounds, of which 19 could not be indentified, were found. Alanine, aspartic acid, cystine, γ-aminobutyric acid, glutamic acid, glutamine, glycine, phenylalanine, serine, threonine, tyrosine and valine occurred in most or all of the species examined. Pipecolic acid was identified in some species of *Protea* only. *Leucadendron* was the only genus containing citrulline in addition to arginine and ornithine; the urea cycle seems to operate in this genus. Although the unidentified compounds were usually present in relatively small amounts, three of them (U–3, U–4 and U–7) were of major importance in those species of *Protea* and *Faurea* in which they were found.

With the exception of the occurrence of citrulline, arginine and ornithine in *Leucadendron*, the distribution of ninhydrin-positive compounds seems to have little taxonomic significance.

Introduction

The use of paper chromatography has led to the discovery and identification of many amino acids and related compounds in plants, e.g., pipecolic acid (Zacharius, Thompson and Steward, 1954), γ-methylene glutamine and γ-methylene glutamic acid (Zacharius, Pollard and Steward, 1952), and azetidine-2-carboxylic acid (Fowden, 1955). Many substances have not been identified, however. In 1955, Steward, Zacharius and Pollard recorded the occurrence of 85 known and 84 unidentified nitrogenous compounds in nature.

Systematic chromatographic surveys have been made of the nitrogenous compounds in certain plant groups, e.g., hops (Harris and Tatchell, 1953), the marine algae (Coulson, 1953), and the Liliaceae (Fowden and Steward, 1957). Some of these surveys have supplied information regarding the possible occurrence of new or unusual metabolic pathways (Fowden and Steward, 1957). The information obtained in this way has also been used to study possible chemical taxonomic relationships.

TABLE 1.

List of species examined

Ref. No.	*Species*
Br–1	Brabeium stellatifolium Linn.
Pa–1	Paranomus crithmifolia R. Br.
Pa–2	P. reflexa Phillips & Hutchinson.
Se–1	Serruria artemisiaefolia Knight.
Se–2	S. burmanni R. Br.
Se–3	S. florida Knight.
Mi–1	Mimetes lyrigera Knight.
Fa–1	Faurea magnaughtonii Phillips.
Pr–1	Protea acaulis Thunb.
Pr–2	P. arborea Houtt.
Pr–3	P. barbigera Meisn.
Pr–4	P. cedromontana Schlechter.
Pr–5	P. compacta R. Br.
Pr–6	P. cordata Thunb.
Pr–7	P. cynaroides Linn.
Pr–8	P. grandiceps Tratt.
Pr–9	P. incompta R. Br.
Pr–10	P. lanceolata E. Mey.
Pr–11	P. eximia (Salisb. ex Knight) Fourc. (P. latifolia R. Br.).
Pr–12	P. laurifolia Thunb.
Pr–13	P. longiflora Lam.
Pr–14	P. nana Thunb.
Pr–15	P. neriifolia R. Br.
Pr–16	P. obtusifolia Buek.
Pr–17	P. odorata Thunb.
Pr–18	P. repens (Linn.) Linn. non Thunb.
Pr–19	P. scolymocephala Reichard.
Pr–20	P. susannae Phillips.
Ls–1	Leucospermum attenuatum R. Br.
Ls–2	L. candicans Loud.
Ls–3	L. catherinae Compton.
Ls–4	L. conocarpum R. Br.
Ls–5	L. linearé R. Br.
Ls–6	L. murii Phillips.
Ls–7	L. nutans R. Br.
Ls–8	L. patersonii Phillips.
Ls–9	L. prostratum Stapf.
Ls–10	L. oleaefolium R. Br.
Ls–11	L. reflexum Buek.
Ld–1	Leucadendron adscendens R. Br.
Ld–2	L. argenteum R. Br.
Ld–3	L. concinnum R. Br.
Ld–4	L. daphnoides Meisn.
Ld–5	L. decorum R. Br.
Ld–6	L. discolor Buek.
Ld–7	L. grandiflorum R. Br.
Ld–8	L. salignum R. Br.
Ld–9	L. sericocephalum Schlechter.
Au–1	Aulax cneorifolia Knight.
Au–2	A. pinifolia Berg.

The Proteaceae constitute an important South African family of the flowering plants. Relatively little is known about their chemical composition, particularly in regard to soluble nitrogenous constituents. The occurrence and distribution of some soluble nitrogenous compounds in a number of species of the Proteaceae were therefore investigated. It was also hoped to determine whether the distribution of these substances might be of any taxomic significance.

MATERIAL AND METHODS

Fifty species, belonging to nine genera of the Proteaceae, viz., *Brabeium*, *Paranomus*, *Serruria*, *Mimetes*, *Faurea*, *Protea*, *Leucospermum*, *Leucadendron* and *Aulax*, were examined. The plants were collected and identified at the National Botanic Gardens, Kirstenbosch. The different species, together with their reference numbers, are listed in Table 1. The genera are arranged according to the classification of Engler and Prantl (1893).

Healthy, approximately one-year-old leaves were collected on the 25th February, 1964, from plants growing in the National Botanic Gardens, Kirstenbosch. The leaves were put in polyethylene bags and stored at -9°C until required for analysis. The soluble nitrogenous fraction was obtained as follows: Ten grams of leaf material were macerated in a Waring blendor with sufficient absolute ethanol to give a final concentration of 80% ethanol. The homogenate was filtered and the residue washed with an additional 100 ml 80% ethanol. Pigments and lipids were removed by shaking the combined filtrate with three volumes of chloroform and separating off the aqueous layer, containing the soluble nitrogenous fraction (Dörfel, 1958). Amino acids and amides were removed from the aqueous extract by passing it through a column of Amberlite IR–120 resin (H^+ form), washing the column with deionised water and eluting them from the resin with 2N NH_4OH until the eluate gave a negative test with ninhydrin. The eluate was concentrated to dryness *in vacuo* at a temperature below 40°C and the residue dissolved in two ml 10% isopropanol.

A 350 μl aliquot of each extract was chromatographed two-dimensionally on Whatman No. 1 chromatography paper, with phenol:water (8 : 2; v/v, adjusted to pH 5·5 with NaOH) in the first (short) and n-butanol:acetic acid: water (4:1:1; v/v) in the second dimension for 22 hours. The chromatograms were dried, sprayed with 0·25% ninhydrin in acetone, and finally dried at 60°C. During the development of the chromatograms the temperature in the laboratory ranged between 18 and 29°C. Although it affected the chromatograms, the separations were always sufficient to distinguish among the different compounds.

The above procedure was employed for the separation and identification of ninhydrin-positive substances as well as for the estimation of their relative concentrations. These were judged according to the relative sizes and intensities of the ninhydrin-reacting spots, and were expressed in terms of an arbitrary scale from one to five, ranging from a trace upwards (see Tables 2 to 5). Wherever necessary, the ninhydrin-reacting spots were identified by means of

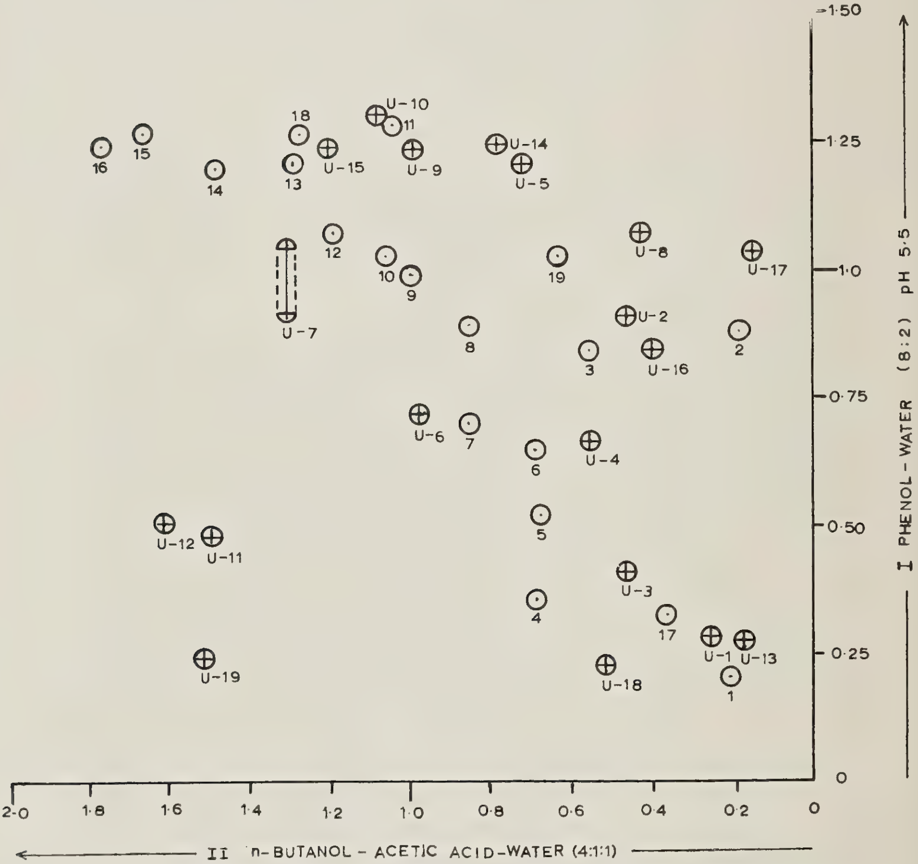

FIG. 1. Positions of free ninhydrin-positive compounds found in the Proteaceae (R-alanine values indicated along abscissa and ordinate):
1—cystine; 2—arginine; 3—glutamine; 4—aspartic acid; 5—serine; 6—glycine; 7—glutamic acid; 8—threonine; 9—alanine; 10—β-alanine; 11—proline; 12—tyrosine; 13—γ-aminobutyric acid; 14—valine; 15—phenylalanine; 16—leucine/isoleucine; 17—ornithine; 18—pipecolic acid; 19—citrulline; U-1 to U-19—unidentified substances. ⊙ — identifiable compounds; ⊕ — unidentified compounds.

TABLE 2.

Relative concentrations of ninhydrin-positive compounds in Brabeium, Paranomus, Serruria, Mimetes, Faurea and Aulax*

	Species Reference No. **									
Compound	Br–1	Pa–1	Pa–2	Se–1	Se–2	Se–3	Mi–1	Fa–1	Au–1	Au–2
Alanine	5	4	5	4	4	4	3	4	4	3
Arginine	–	–	–	–	–	–	–	1	–	–
Aspartic acid	2	3	3	4	2	4	3	2	3	2
β-alanine	–	1	1	–	1	1	–	–	–	–
Cystine	1	2	2	2	1	2	2	1	2	2
γ-aminobutyric acid	4	4	4	4	4	4	3	5	4	4
Glutamic acid	4	5	4	5	5	4	5	4	5	5
Glutamine	2	2	3	2	2	3	2	2	3	1
Glycine	2	3	3	4	3	3	3	3	3	2
Leucine/isoleucine	–	–	1	–	–	–	–	–	–	–
Phenylalanine	2	2	2	2	1	2	1	2	2	1
Proline	–	–	–	1	–	–	–	–	–	–
Serine	3	3	3	4	3	4	3	3	4	3
Threonine	2	2	2	2	2	3	2	2	2	1
Tyrosine	3	2	–	2	1	2	2	3	1	–
Valine	2	2	2	2	2	2	2	2	2	1
Unidentified U–1	–	–	–	–	–	–	–	–	–	2
U–2	–	–	–	–	–	–	–	–	1	–
U–3	–	–	–	–	–	–	–	3	–	–
U–4	–	–	–	–	–	–	–	3	–	–
U–5	–	1	1	1	1	1	–	–	–	–
U–6	–	–	–	–	–	–	–	–	2	–
U–7	–	–	–	–	–	–	–	4	–	–

*Relative concentrations: 1 = Trace reaction; 2 = Weakly reacting; 3 = Moderately reacting; 4 = Strongly reacting; 5 = Major constituent.

**See Table 1.

TABLE 3.

Relative concentrations of ninhydrin-positive compounds in different species of Protea*

	Species Reference No. Pr**																			
Compound	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Alanine	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	5	4	4
Arginine	–	1	–	–	–	–	–	–	–	2	–	–	–	1	–	–	–	–	1	–
Aspartic acid	3	2	3	3	4	2	3	3	3	3	3	2	4	4	3	3	3	2	3	3
β-alanine	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cystine	1	1	1	1	2	1	2	2	1	2	1	1	2	2	1	2	1	1	2	1
γ-aminobutyric acid	4	3	4	4	3	4	3	4	3	4	4	4	4	4	4	4	3	4	3	4
Glutamic acid	4	5	4	4	5	5	5	5	5	5	4	5	5	5	5	4	5	4	5	5
Glutamine	2	1	2	2	1	3	1	2	1	3	2	1	3	1	1	1	3	2	2	1
Glycine	3	3	2	3	3	1	2	3	2	3	3	2	3	2	3	2	3	3	3	2
Leucine/isoleucine	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ornithine	–	1	–	1	2	–	2	2	–	–	–	–	–	–	–	2	–	–	–	1
Phenylalanine	1	1	2	1	2	2	2	1	2	2	1	1	1	2	1	1	2	1	1	1
Pipecolic acid	–	2	2	–	–	4	2	2	3	3	–	–	–	1	–	2	–	–	–	–
Serine	3	3	3	3	3	3	3	3	3	4	3	3	4	3	3	3	4	3	3	4
Threonine	2	2	1	2	2	2	2	1	2	3	2	2	3	1	2	2	3	2	2	2
Tyrosine	3	3	1	2	–	2	3	3	3	3	2	1	1	3	–	3	2	2	2	1
Valine	2	1	2	1	2	2	2	1	1	2	1	2	1	2	1	1	2	1	2	1
Unidentified: U–3	4	–	–	–	–	4	4	4	–	–	–	–	–	4	–	4	–	–	3	–
U–4	4	–	–	–	–	4	4	4	–	–	–	–	–	4	–	4	–	–	3	–
U–7	5	5	5	–	–	–	5	5	–	–	–	–	–	–	–	5	5	–	–	–
U–8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–
U–9	–	2	2	–	–	–	2	–	–	2	–	–	–	–	–	2	–	–	–	–
U–10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–
U–11	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
U–12	–	–	2	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–

*See subscript to Table 2.

**See Table 1.

co-chromatography or specific spray reagents (Block, Durrum and Zweig, 1958). Where compounds could not be positively identified, they were numbered and their R-alanine values in each of the solvents noted, along with the colour produced with ninhydrin.

RESULTS

The positions of the different ninhydrin-reacting compounds observed on the chromatograms are shown in figure 1. Each spot represents the average of all R-alanine values obtained for the particular compound. The relative concentrations of the various ninhydrin-positive substances found in the different species are listed in Tables 2 to 4. Their frequencies in the different genera are summarised in Table 5. The ninhydrin colours and R-alanine values of the unidentified compounds are given in Table 6.

Altogether 38 ninhydrin-positive substances were found; 19 of these could not be identified. Alanine, γ-aminobutyric acid, aspartic acid, glutamine and serine were present in extracts from all species in moderate to relatively high amounts. Glutamic acid tended to be the major free amino acid. Cystine,

TABLE 4.

Relative concentrations of ninhydrin-positive compounds in species of Leucospermum and Leucadendron*

	Species Reference No.**																			
	Leucospermum (Ls)											Leucadendron (Ld)								
Compound	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	8	9
Alanine	4	4	4	4	4	5	4	5	5	4	5	4	4	4	4	3	4	4	4	4
Arginine	–	1	–	–	–	–	1	1	2	1	–	1	2	1	1	1	1	1	2	1
Aspartic acid	3	3	3	3	3	3	3	3	4	4	2	4	3	3	3	4	2	3	4	4
β-alanine	–	1	1	–	–	1	1	1	1	2	1	–	–	–	–	–	–	–	–	–
Citrulline	–	–	–	–	–	–	–	–	–	–	–	–	2	3	–	3	1	1	1	1
Cystine	1	2	2	1	2	1	2	2	1	1	1	2	2	2	2	3	3	2	2	2
γ-aminobutyric acid	4	4	4	5	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Glutamic acid	5	5	4	4	5	4	5	3	4	4	4	4	4	4	4	4	4	4	4	4
Glutamine	2	2	4	2	2	2	2	2	2	4	2	1	2	2	1	2	1	2	1	2
Glycine	3	2	3	3	3	3	3	2	2	3	3	2	2	2	2	3	3	2	2	2
Leucine/isoleucine	–	–	2	–	2	–	–	2	2	–	–	–	–	–	–	–	–	–	–	–
Ornithine	–	–	–	–	–	1	–	–	–	–	–	2	1	2	2	3	2	1	2	1
Phenylalanine	1	2	2	2	2	2	2	2	2	2	2	–	3	2	–	2	1	2	3	2
Proline	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Serine	2	3	4	4	4	3	3	3	3	3	2	3	4	2	2	4	3	4	2	2
Threonine	2	3	2	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3
Tyrosine	–	1	–	1	3	1	1	–	–	–	–	2	3	2	1	3	1	2	2	2
Valine	2	2	2	2	2	2	2	2	2	2	2	2	2	2	–	1	1	1	1	2
Unidentified: U–5	–	1	–	–	–	1	1	1	–	–	1	–	–	–	–	–	–	–	–	–
U–8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–	1	2	1
U–13	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
U–14	–	–	1	–	1	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–
U–15	–	1	–	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–
U–16	–	–	–	–	2	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–
U–17	–	–	–	–	–	–	–	–	–	–	–	1	1	1	–	1	1	1	1	1
U–18	–	–	–	–	–	–	–	–	–	–	–	–	2	2	2	–	1	3	3	3
U–19	–	–	–	–	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	–

* See subscript to Table 2.
** See Table 1.

TABLE 5.

Frequency percentages of different ninhydrin-positive substances in different genera of the Proteaceae

Compound		Brabeium*	Paranomus	Serruria	Mimetes*	Faurea*	Protea	Leuco-spermum	Leuca-dendron	Aulax
Alanine		100	100	100	100	100	100	100	100	100
Arginine		0	0	0	0	100	20	45	100	0
Aspartic acid		100	100	100	100	100	100	100	100	100
β-alanine		0	100	67	0	0	5	73	0	0
Citrulline		0	0	0	0	0	0	0	78	0
Cystine		100	100	100	100	100	100	100	100	100
γ-aminobutyric acid		100	100	100	100	100	100	100	100	100
Glutamic acid		100	100	100	100	100	100	100	100	100
Glutamine		100	100	100	100	100	100	100	100	100
Glycine		100	100	100	100	100	100	100	100	100
Leucine/isoleucine		0	50	0	0	0	5	36	0	0
Ornithine		0	0	0	0	0	35	9	100	0
Phenylalanine		100	100	100	100	100	100	100	78	100
Pipecolic acid		0	0	0	0	0	45	0	0	0
Proline		0	0	33	0	0	0	9	0	0
Serine		100	100	100	100	100	100	100	100	100
Threonine		100	100	100	100	100	100	100	100	100
Tyrosine		100	50	100	100	100	90	45	100	50
Valine		100	100	100	100	100	100	100	89	100
Unknowns:	U–1	0	0	0	0	0	0	0	0	50
	U–2	0	0	0	0	0	0	0	0	50
	U–3	0	0	0	0	100	35	0	0	0
	U–4	0	0	0	0	100	35	0	0	0
	U–5	0	100	100	0	0	0	45	0	0
	U–6	0	0	0	0	0	0	0	0	50
	U–7	0	0	0	0	100	35	0	0	0
	U–8	0	0	0	0	0	5	0	44	0
	U–9	0	0	0	0	0	25	0	0	0
	U–10	0	0	0	0	0	5	0	0	0
	U–11	0	0	0	0	0	5	0	0	0
	U–12	0	0	0	0	0	10	0	0	0
	U–13	0	0	0	0	0	0	9	0	0
	U–14	0	0	0	0	0	0	36	0	0
	U–15	0	0	0	0	0	0	27	0	0
	U–16	0	0	0	0	0	0	27	0	0
	U–17	0	0	0	0	0	0	0	89	0
	U–18	0	0	0	0	0	0	0	78	0
	U–19	0	0	0	0	0	0	0	11	0

* Only one species examined.

glycine, phenylalanine, threonine, tyrosine and valine occurred in most or all of the extracts, but generally at lower levels than the former group. Three components of the urea cycle were identified: arginine and ornithine occurred in some species of *Protea* and *Leucospermum*, but generally not together in the same species; relatively small amounts of arginine and ornithine were present in all species of *Leucadendron*, and citrulline in most of them. This was the

TABLE 6.

Colour changes and R-alanine values of unidentified ninhydrin-positive substances

Unidentified Compound No.	Ninhydrin colour*	R-alanine values in: Phenol/water	R-alanine values in: Butanol/acetic acid/water
U–1	P	0.28	0.25
U–2	P	0.91	0.46
U–3	Y–P	0.42	0.46
U–4	Y–P	0.67	0.55
U–5	P	1.21	0.72
U–6	P	0.73	0.97
U–7	YB–B–P	0.98	1.31
U–8	P	1.08	0.42
U–9	P	1.24	0.99
U–10	P	1.31	1.08
U–11	P	0.48	1.49
U–12	P	0.51	1.61
U–13	P	0.28	0.17
U–14	P	1.25	0.79
U–15	P	0.28	1.20
U–16	P	0.85	0.40
U–17	P	1.04	0.14
U–18	P	0.17	0.52
U–19	P	0.24	1.51

* Colours: P = purple; YB = yellow-brown; Y = yellow; B = brown.

only genus in which citrulline was observed. Pipecolic acid was identified in a number of *Protea* species only. Glutamine, the only amide identified, was present in low to moderate amounts in all species. Leucine-isoleucine and proline were observed in only a few species.

The unidentified substances were not widely distributed. No unidentifiable compounds were found in *Brabeium stellatifolium* (Br–1), *Mimetes lyrigera* (Mi–1), eight *Protea* and four *Leucospermum* species. One compound, U–5, was found in altogether 10 species of *Paranomus*, *Serruria* and *Leucospermum*. U–8 occurred in one species of *Protea* and four of *Leucadendron*, and U–3, U–4 and U–7 in *Faurea magnaughtonii* and some *Protea* species in fairly large amounts, with the former two always together. The other unidentifiable substances (U–1, U–2, U–6 and U–9 to U–19) were found in only one genus each.

Three of the unidentified compounds, viz., U–3, U–4 and U–7, underwent marked colour changes within 12 hours after spraying with ninhydrin: U–3 and U–4 changed from yellow to purple, and U–7 from yellowish-brown through brown to purple. Judging from the intensity of the ninhydrin colour and spot size, U–7 appeared to be the major free ninhydrin-positive compound in those species in which it was present.

DISCUSSION

Although some 50 species of the Proteaceae were analysed, no specific distribution pattern of ninhydrin-positive substances could be recognised for the family as such. Practically all the species examined contained alanine, aspartic acid, cystine, γ-aminobutyric acid, glutamic acid, glutamine, glycine, phenylalanine, serine, threonine and valine in varying amounts. It is doubtful, however, whether this composition would distinguish the Proteaceae from other families.

When one considers the individual genera, *Leucadendron* differs from the rest in that it is the only genus containing citrulline; in addition, it is the only genus in which three components of the urea cycle, viz., arginine, citrulline and ornithine, occur fairly consistently. The two unknown compounds U–17 and U–18, although present in very small amounts, might also be typical of *Leucadendron*.

Although arginine and ornithine occur in *Protea*, their frequencies are too low for them to be regarded as distinctive. The fact that pipecolic acid was found in some *Protea* spp. is of interest, but because of its low frequency it cannot be used to distinguish *Protea* from other genera of this family. Pipecolic acid might have been present in some other genera and species, because its identification is complicated by the fact that it does not separate completely from γ-aminobutyric acid.

Although the relatively high concentrations of the unknowns U–3, U–4 and U–7 in *Protea* and *Faurea* make them interesting from a biochemical viewpoint, their presence is of doubtful taxonomic value because of their low frequencies in *Protea* and the fact that only one species of *Faurea* was analysed. The other unknown substances occurred in only one genus each, but with the exception of U–17 and U–18 in *Leucadendron*, their distribution was too sporadic or too few species were examined for them to be of any taxonomic value.

It would appear, therefore, that with the exception of the components of the urea cycle (particularly citrulline) found in *Leucadendron*, no particular and consistent distribution pattern of free ninhydrin-reacting compounds could be observed in the Proteaceae. The occurrence of arginine, citrulline and ornithine in *Leucadendron* is of both biochemical and taxonomic interest, but more species will have to be examined and attempts made to identify the unknown compounds, before a complete picture of the free amino acids in the Proteaceae and their taxonomic significance (if any) can be obtained. The possibility that nutritional and environmental factors may affect the occurrence and concentration of these compounds must also be considered.

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